

The Means To An End: Optimal Methods for DNA Fragmentation and End Repair

Thomas Schoenfeld, Ronald Godiska, Melodee Patterson, and David Mead
Lucigen Corporation

Introduction

Random shotgun sequencing is the typical method used to determine the sequence of a genome. The success and efficiency of this process is dependent on random fragmentation of the DNA and unbiased cloning of these fragments to generate a shotgun library. An “ideal” library would provide even, unbiased coverage, yield a sufficient number of clones for sequence overlap, and have no background of clones without inserts. Lucigen has developed a transcription-free cloning system that improves library construction by eliminating several forms of cloning bias and dramatically reducing vector background (1). This technology is incorporated into the CloneSmart® and ClonePlex® kits, which provide all the components needed for library construction except the insert DNA.

Careful preparation of the insert DNA fragments is essential for successful construction of a shotgun library. Preparation of random fragments for shotgun cloning includes: 1) DNA isolation, 2) fragmentation, 3) end repair, and 4) size fractionation. The research presented here is directed at optimizing these steps.

Isolation of DNA

Regardless of the source of insert DNA, the purity, quantity, and integrity of the sample is critical to the success of the library construction. A common cause of failure is over-estimating the quantity or the integrity of the input DNA. These factors should be verified prior to beginning the process of library construction.

Shearing

Most large-scale sequencing strategies depend on random fragmentation of the target into smaller pieces for sub-cloning. Physical fragmentation methods such as nebulization (2), sonication (3), and hydrodynamic shearing (e.g., HydroShear™ by GeneMachines™) (4) are generally preferred over enzymatic methods, because they are more random, more readily controlled, and they result in a collection of overlapping fragments.

Bacteriophage lambda DNA sheared by these three physical methods is shown in Fig. 1. While all three methods effectively sheared the DNA, HydroShear fragmentation resulted in a narrower size range than either of the other methods. The settings on the HydroShear instrument can be adjusted to modulate the size range from 500 bp to >10,000 bp. In this experiment, 86% of the HydroShear DNA (hs5) was in the desired size range of 1 to 3 kb, compared to 65% and 56% for nebulized and sonicated DNA, respectively. As a result, less starting material was required when using the HydroShear apparatus.

A comparison of other aspects of the shearing methods is shown in Table 1. In general, nebulization and sonication are more affordable or accessible to many labs, whereas HydroShear fragmentation appears to produce the highest quality and quantity of DNA fragments.

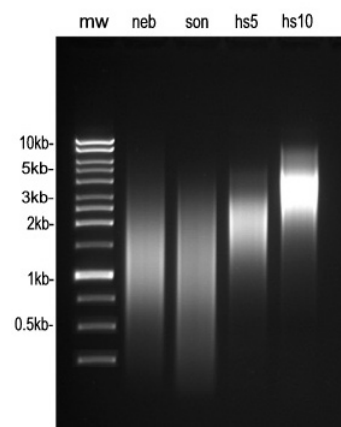


Figure 1. Comparison of Shearing Methods. Lambda DNA (5 µg) was sheared by nebulization (neb), sonication (son), or hydrodynamic fragmentation (hs5 or hs10). The nebulization parameters were: volume = 2 ml, pressure = 20 psi, time = 2.5 minutes. Sonication was performed with a probe type Branson Sonifier at the following settings: volume = 400 µl, power = 1, duty = 100%, time = 2 cycles of 3 sec. The HydroShear parameters were: volume = 200 µl, cycles = 20, speed = 5 or 10 (indicated by “hs5” or “hs10”, respectively). Five hundred ng of each sample was electrophoresed in a 1% agarose/TAE gel.

	Nebulization	Sonication	HydroShear™
Advantages	<ul style="list-style-type: none"> • Low start up costs • Rapid 	<ul style="list-style-type: none"> • Fast • Easy • Low risk of carryover 	<ul style="list-style-type: none"> • Size-specific • Narrow size range • Less DNA damage
Disadvantages	<ul style="list-style-type: none"> • Large Volume • DNA must be concentrated • Potential for DNA carryover • Equipment modification 	<ul style="list-style-type: none"> • Not size-specific • DNA is damaged • Expensive equipment 	<ul style="list-style-type: none"> • Expensive equipment • More complicated, time consuming process

Continued on page 3 **Table 1. Advantages and disadvantages of DNA shearing methods.**

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End Repair

Physical shearing methods produce fragments having heterogeneous ends with 5' or 3' overhangs of varying lengths. These ends must be treated to produce blunt ends that are effective substrates for the ligation reaction. Various enzymatic treatments can be used to generate blunt ends. Single-strand specific exonucleases, particularly mung bean nuclease, are used to digest the single stranded regions, leaving blunt ends. Alternatively, blunt ends can be produced by T4 DNA polymerase, which has a potent single-strand exonuclease that removes 3' overhangs and a DNA polymerase activity that fills in 5' overhangs. In some protocols the T4 DNAP is augmented by Klenow fragment of *E. coli* DNA polymerase I. The DNATerminator® kit uses a proprietary mix of enzymes to produce blunt ends.

To compare these protocols, phage lambda DNA was sheared by nebulization, sonication, or HydroShear™ fragmentation. The samples were subsequently treated by each of the end-repair methods, cloned using the ClonePlex® or CloneSmart® systems, and tested for the number of resulting transformants. Nebulization and mung bean nuclease treatments resulted in 50-75% fewer transformants (data not shown). DNATerminator end repair was compared to T4 DNAP plus Klenow treatment using size selected, sonicated, and HydroShear treated DNA as a substrate (Fig. 2). For the more demanding application of dual-insert cloning, use of the DNATerminator Kit resulted in 5-fold more colonies from sonicated DNA and 2-fold more colonies from either HydroShear treated sample (1-3 kb HS5 or 2-5 kb HS10). The DNATerminator end repair reaction also provided a clear advantage for single-insert cloning of larger fragments. Both methods worked well for cloning inserts of 1-3 kb.

Size fractionation

Even if most of the sheared DNA is in the desired size range, transformation of *E. coli* cells strongly selects for trace amounts of small fragments. For this reason, it is highly recommended that sheared, end repaired DNA be size selected by electrophoresis in low melting agarose gels. This method has the added benefit that it effectively removes residual enzymes and other components of the end repair reactions. If the size selection step is omitted, it is necessary to use a DNA cleanup step such as phenol/chloroform or a commercial kit. After size fractionation, it is critical to quantify the resulting DNA by gel electrophoresis with the appropriate mass and molecular weight standards. A common cause of low transformation efficiency is over-estimating the amount of insert added to the ligation reaction.

Recommended protocol for preparation of insert DNA

Based on these results, we recommend the following protocol for optimal construction of large shotgun libraries:

- 1) Verify the quantity and quality of the starting DNA.
- 2) Shear the DNA to the desired molecular weight using the HydroShear apparatus (GeneMachines™, San Carlos, CA). Sonication or nebulization result in lower quality fragments.
- 3) End repair the insert DNA using the DNATerminator Kit according to the instructions.
- 4) Size-fractionate the DNA by electrophoresis in a low melting temperature agarose gel to remove low molecular weight DNA and other contaminants.
- 5) Purify the DNA from the gel using standard techniques.
- 6) Quantify the DNA by agarose gel electrophoresis with a reliable mass standard.
- 7) Clone the insert into pSMART or pLEXX™-AK according to the CloneSmart or ClonePlex Manual.

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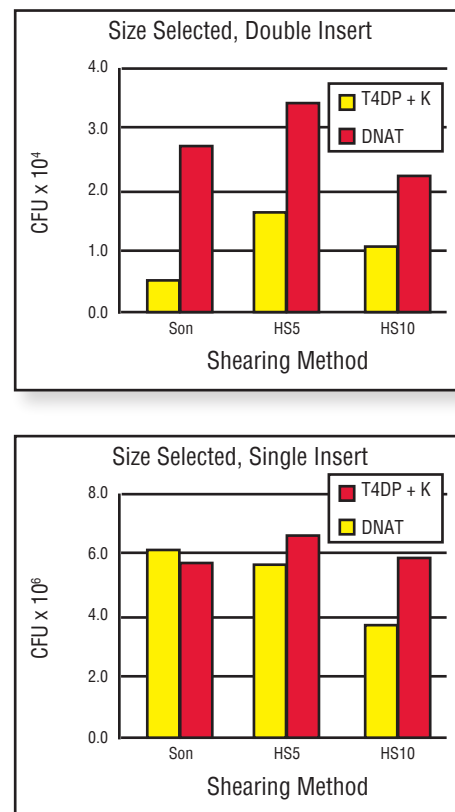


Figure 2. End Repair of Size Selected Sheared DNA. Sonicated and HydroShear fragmented DNA samples (Fig. 1) were end repaired either with T4 DNA polymerase + Klenow (T4DP + K) or with the DNATerminator Kit (DNAT). Gel electrophoresis was used to select fragments of 1-3 kb (Son and HS5) or 2-5 kb (HS10). These fragments were inserted into the dual-insert ClonePlex vector (upper panel) or the single-insert CloneSmart vector (lower panel).

Means To An End...Cont.

Use of this protocol typically results in libraries of >1,000,000 single insert clones or >10,000 dual insert clones with less than 0.1% empty vector background.

References

1. Godiska R, Reuter M, Schoenfeld T, Sheets L, Derr A, and Mead D 2001. *eLucidations* 1: 1-3.
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3. Deininger PL 1983. *Anal. Biochem.* 129: 216-223.
4. Thorstenson YR, Hunicke-Smith SP, Oefner PJ, Davis RW 1998. *Genome Res.* 8:848-55.

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Product Information

	Size	Electrocompetent Cells		Chemically Competent 10G Cells	w/o Cells	
		10G ELITE	10G SUPREME		Size	Cat.#
CloneSmart® HC Blunt Cloning Kits		Cat.#	Cat.#	Cat.#		
High copy number pSMART™ HCAmp Vector Premix	10 libraries	40052-1	40063-1	40074-1	20 rxns	40041-2
	20 libraries	40052-2	40063-2	40074-2	40 rxns	40041-4
High copy number pSMART HCKan Vector Premix	10 libraries	40706-1	40717-1	40728-1	20 rxns	40704-2
	20 libraries	40706-2	40717-2	40728-2	40 rxns	40704-4
CloneSmart LC Blunt Cloning Kits						
	Low copy number pSMART LCAmp Vector Premix	10 libraries	40311-1	40322-1	40333-1	20 rxns
20 libraries		40311-2	40322-2	40333-2	40 rxns	40300-4
Low copy number pSMART LCKan Vector Premix	10 libraries	40832-1	40843-1	40854-1	20 rxns	40821-2
	20 libraries	40832-2	40843-2	40854-2	40 rxns	40821-4

All **CloneSmart** Kits include CloneSmart Ligase, two sequencing primers, positive control insert DNA, and positive control transformation plasmid.

	Size	Cat.#
DNATerminator® End Repair Kit End repair buffer, end repair enzyme mix	10 transformations	40035-1
	50 transformations	40035-2

Construction of cDNA Libraries...Cont.

The CloneSmart Blunt Cloning Kit is certainly a very quick and easy system to use, requiring a much shorter time to generate libraries, which contain numerous previously elusive clones. With the CloneSmart Kit we now have large fully represented cDNA libraries – a simple remedy with a significant result! We will certainly continue with the CloneSmart system and recommend it highly.

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Xenome Ltd.
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phone: 888-575-9695
608-831-9011

fax: 608-831-9012

e-mail: lucigen@lucigen.com

web: www.lucigen.com

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